

# **Development of individualized surgical treatments for malignant melanoma**

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Gothenburg, Sweden  
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To my Family

“Per aspera ad astra.”  
Cicero (De natura deorum III, 40)

# **Development of individualized surgical treatments for malignant melanoma**

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**Background:** Cutaneous melanoma is a malignancy with an increasing incidence worldwide, especially in northern Europe. The aim of this thesis is to scrutinize the results achieved by traditional surgery and the opportunities offered by translational research for the more advanced stages of the disease.

**Paper I** analysed outcomes of sentinel node biopsy (SNB) performed on 769 consecutive patients with cutaneous melanoma. Breslow thickness was the only predictive factor for a positive SN. The 5-year melanoma specific survival (MSS) was 81% and in multivariate analysis the negative prognostic factors for survival were SN-status, followed by Breslow thickness and ulceration.

**Paper II** reported on 290 consecutive patients who underwent 380 isolated limb perfusion (ILP), of which 90 were re-ILPs. The results between the 1st, the 2nd and the 3-5th were compared. Patients with a complete response at the first treatment were likely to have the same response at re-ILP without any increase in the risk for local toxicity or complications.

**Paper III** BRAF mutational status as a predictive factor for response was studied in 98 patients who underwent ILP. In this consecutive series, 32 patients had a BRAF V600E/K mutation and 66 patients were BRAF wild type, and no significant correlation for response or survival was found.

**Paper IV** was a translational study based on patient-derived xenograft models including 21 cutaneous melanoma biopsies transplanted into either NOG or IL-2 transgenic NOG (hIL2-NOG) mice. It was shown

that the models reliably could be used to predict the effect of tumour-infiltrating lymphocytes against the tumours.

**Conclusions:** The surgical approach and therapies for patients with cutaneous melanoma are becoming more targeted and personalized. A specialised multidisciplinary approach can improve the understanding of the disease, support the decision-making process towards the most advantageous treatment options for each individual patient at a specific time.

**Keywords:** Melanoma, sentinel node, isolated limb perfusion, immune-humanized xenograft mouse models, translational research.

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# **SAMMANFATTNING PÅ SVENSKA**

## **Bakgrund**

Malignt melanom är en hudcancer med ökande förekomst över hela världen, särskilt i norra Europa. Syftet med denna avhandling var att studera kirurgiska metoder och kombinationen mellan experimentell kirurgi och translationell forskning för att behandla de mer avancerade stadierna av sjukdom.

## **Metod**

Kliniska utfall analyserades retrospektivt för patienter som genomgick portvaktsskörtelbiopsi (sentinel node biopsi, SNB) och isolerad hyperterm perfusion (isolated limb perfusion, ILP) på Sahlgrenska Universitetssjukhuset, med särskild inriktning på upprepade behandlingar (re-ILP) och resultat som relaterats till tumörens mutationsstatus. Tumörprover användes dessutom för att skapa en biobank och producera avancerade musmodeller med patientens egna tumörer, så kallade patient-derived xenografts (PDX).

## **Resultat**

**Studie I** inkluderade 769 konsekutiva patienter som genomgått SNB. Tumörtjocklek enligt Breslow var den enda prediktiva faktorn för positiv SN. Melanomspecifik överlevnad vid 5 år var 81% och i multivariatanalys var de viktigaste negativa prognostiska faktorerna SN status, följt av Breslow-tjocklek och förekomst av ulceration i primärtumören.

**Studie II** rapporterade 290 konsekutiva patienter som genomgått totalt 380 stycken ILP, varav 90 var upprepade behandlingar upp till fem gånger på samma patient. Behandlingsresultatet var likvärdigt vid upprepade behandling, och de patienter som hade komplett respons vid första behandlingen, hade stor sannolikhet för att uppnå detta även vid upprepade behandling. Det fanns ingen ökad risk för lokal toxicitet eller komplikationer vid upprepade behandling.

**Studie III** studerade 98 patienter som genomgått ILP och BRAF-mutationsstatus analyserades som en prediktiv faktor för respons. Totalt hade 32 patienter en BRAF V600E/K-mutation och 66 patienter var BRAF wild type. Någon signifikant korrelation mellan BRAF-status och respons eller överlevnad kunde dock inte visas.

**Studie IV** var en translationell studie baserad på 21 tumörer som transplanterats till möss som saknar immunförsvar (NOG) eller samma typ av möss som är genetiskt modifierade för att producera mänskligt interleukin-2 (hIL2-NOG). Studien har visat att denna modell kan användas för att förutsäga immunförsvarets aktivitet mot patientens tumör, och att detta därför i framtiden skulle kunna ge en vägledning för om patienten kommer att svara på immunterapi eller inte.

### **Slutsats**

Behandlingen av malignt melanom blir allt mer precis och kliniska studier gör att vi bättre kan rekommendera patienterna lämplig behandling. Möjligheten till att använda avancerade musmodeller av patienternas egna tumörer kan även underlätta skräddarsydd behandling i en nära framtid.



## LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals (I-IV).

- I. Belgrano V., Katsarelis D., Mattsson J., Olofsson Bagge R.  
**Sentinel node for malignant melanoma: An observational study of a consecutive single centre experience.**  
Eur J Surg Oncol. 2019 Feb;45(2):225-230
- II. Belgrano V., Pettersson J., Nilsson J.A., Mattsson J., Katsarelis D., Olofsson Bagge R.  
**Response and toxicity of repeated isolated limb perfusion (re-ILP) for patients with in-transit metastases of malignant melanoma.**  
Ann Surg Oncol. 2019 Apr;26(4):1055-1062
- III. Belgrano V., Mattsson J., Nilsson J.A., Olofsson Bagge R., Katsarelis D.  
**BRAF status as a predictive factor for response in isolated limb perfusion.**  
Int J Hyperthermia. 2019 Apr;36(1):511-515
- IV. Ny L., Rizzo L., Belgrano V., Karlsson J., Jespersen H., Carstam L., Olofsson Bagge R., Nilsson L.M. and Nilsson J.A.  
**Supporting clinical decision-making in advanced melanoma by preclinical testing in personalized immune-humanized xenograft mouse models.** Manuscript submitted.

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## **ABBREVIATIONS**

AJCC	American Joint Committee on Cancer
ALM	Acral lentiginous melanoma
BAC	Best alternative care
CLND	Completion lymph node dissection
CT	Computed tomography
DFS	Disease-free survival
EGFR	Epidermal growth factor receptor
HLA	Human leukocyte antigen
ICD	International Classification of Diseases
ICU	Intensive care unit
ILI	Isolated limb infusion
ILP	Isolated limb perfusion
LMM	Lentigo maligna melanoma
M-ILP	Melphalan based isolated limb perfusion
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging

NM	Nodular melanoma
NSN	Non sentinel node
OS	Overall survival
PCR	Polymerase chain reaction
PD	Progressive disease
PET-CT	Positron emission tomography – computed tomography
PFS	Progression-free survival
PR	Partial response
RECIST	Response Evaluation Criteria In Solid Tumours
RNA	Ribonucleic acid
SD	Stable disease
SN	Sentinel node
SNB	Sentinel node biopsy
SSM	Superficial spreading melanoma
TM-ILP	TNF-alpha and melphalan based isolated limb perfusion
TNF-alpha	Tumour necrosis factor-alpha
TTLP	Time to local progression
UV	Ultraviolet light
WHO	World Health Organization



# **1. INTRODUCTION**

## **1.1. Melanocyte biology**

Melanocytes have a common origin deriving from neural crest cells, they migrate during the embryogenic process as melanoblasts and they reach their final destination in the body (skin, conjunctiva, uvea, inner ear, brain, heart) where they achieve their mature state [1]. All melanocytes have similar biology and basic function with production of melanin through a chemical process completed by tyrosinase enzymes in the melanosomes. Either eumelanin or pheomelanin is produced, depending on the presence or absence of cysteine and glutathione in the process [2].

The mechanism of cellular melanin production and the following transfer to the surrounding keratinocytes have an UV radiation protective purpose within the skin and conjunctiva [3, 4]. However, the role of melanocytes is still far from being understood in other parts of the body; other functions that have been proposed, include the involvement in hearing and equilibrium functions at the level of the inner ear [5-7], a neuroendocrine and anti-oxidative function in the brain [8] and mechanic and electrical conductive functions in the heart [9, 10].

## **1.2. Epidemiology**

Melanomas derive from the cancerogenic proliferation of melanocytes, but different types of melanoma are caused by different genetic mutations with different incidence, localization and clinical characteristics. Cutaneous melanoma is the most frequent encountered form, with an incidence of 288,000 new cases per year (3.8 per 100,000) as reported by the GLOBOCAN registry in 2018 [11]. Sweden has the 4th highest incidence in Europe, with 40 per 100,000 affected men and 43 per 100,000 women, progressively increasing year by year [12]. Australia has the highest incidence in the world with a reported incidence of 58 per 100,000 for the whole country and a peak of 67 per 100,000 in Queensland [13]. The incidence is similar worldwide between men and women, even if the localization is different. Women more often present with melanomas on the lower extremities, while it is more common with trunk melanomas in men [14].

### **1.3. Subtypes**

In 1969 skin melanoma was classified by Clark from a pathologic point of view [15]. The superficial spreading melanoma (SSM) is typically characterized by two different growth phases, first the radial phase and then the vertical phase. Nodular melanoma (NM) usually shows an immediate tendency to a vertical growth with a more aggressive metastatic behaviour. The lentigo maligna (LM), frequently associated with extensive sun exposure and more often affecting elderly patients, is characterized by a slow proliferation and infrequent spreading behaviour. Acral melanoma (AM) typically has a palmar or plantar localization [15].

### **1.4. Prognostic factors**

The most important prognostic factors are Breslow thickness, ulceration and sentinel node (SN) status [16]. The thickness of the tumour lesion according to Breslow, reported in millimetres, is the most important parameter in terms of survival. Following Breslow criteria, this value is established by measuring the distance between the highest point of the granular layer and the deepest level of infiltration of the tumour. This kind of measurement is reliable only when sections are cut perpendicular to the epidermal layer [17]. Ulceration of the primary tumour was reported as an important prognostic factor by Allen and Spitz in 1953. They describe this condition as the interruption of continuity in a portion of the epidermidis layer [18].

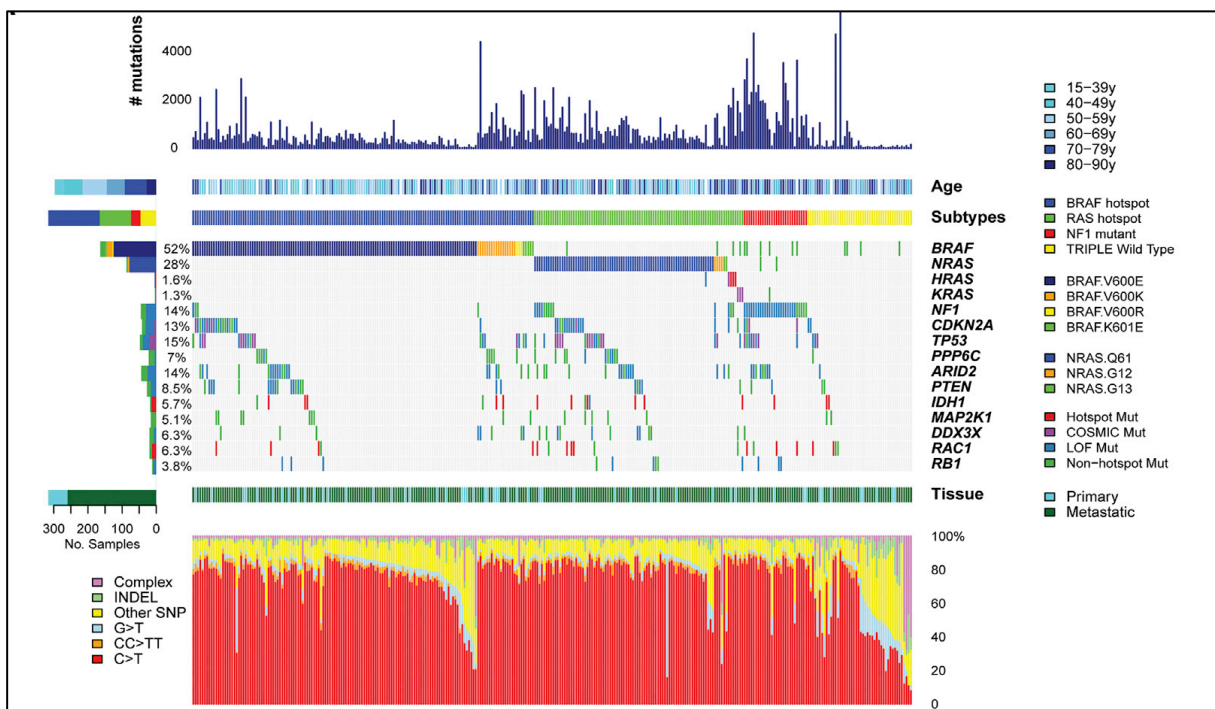
Previously Clark's level of invasion together with mitotic rate, were included in the staging system, however, both these parameters were removed in the latest update, but it is still recommended that these parameters are reported in the pathology report. Clark's levels of tumour invasion are: level I is confined to the epidermidis (in situ), level II is the invasion of the papillary dermis, level III is the involvement of the junction between papillary and reticular layer, level IV is the invasion of the reticular layer and level V is the presence of tumour in the subcutaneous fat [15]. The number of mitosis is reported as significant if in excess of 1 per mm<sup>2</sup> and is considered a direct sign of proliferation activity [19, 20].



## 1.5. Genetic classification

From a genetic perspective, cutaneous melanoma is divided based on mutational status and is grouped into four categories (Figure 1).

1. *BRAF* mutant (v-raf murine sarcoma viral oncogene homolog b)
2. *RAS* mutant (neuroblastoma RAS viral v-ras oncogene homolog)
3. *NF1* mutant (neurofibromin 1)
4. Triple wild-type



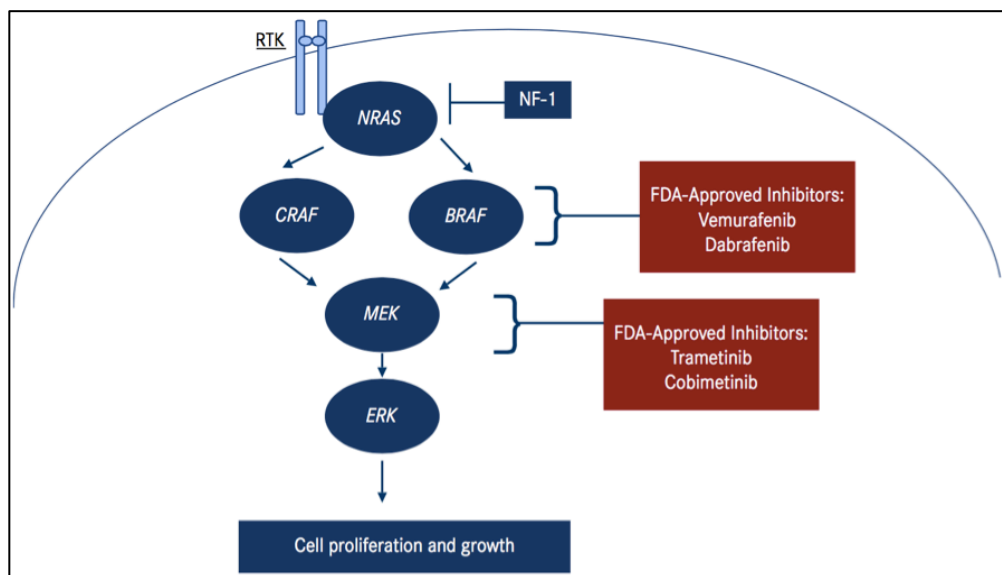
**Figure 1.** Landscape of driver mutations in melanoma: Total number of mutations, age at melanoma diagnosis, and mutation subtype (*BRAF*, *RAS*, *NF1*, and Triple-wild type) are indicated for each sample (top). Color-coded matrix of individual mutations (specific *BRAF* and *NRAS* mutations indicated) (middle), type of melanoma specimen (primary or metastasis), and mutation spectra for all samples (bottom) are indicated. For the two samples with both a matched primary and metastatic sample, only the mutation information from the metastasis was included. Figure adopted from: Genomic Classification of Cutaneous Melanoma. Cancer Genome Atlas Network. Cell. 2015 Jun 18;161(7):1681-96.

These different mutations involve the mitogen-activated protein-kinase (MAPK) pathway with *BRAF* and *NRAS* being oncogenes and *NF1* being a tumour suppressor gene. A mutation in an oncogene produces an uncontrolled signal of proliferation, while a mutation in a tumour suppressor compromises their function as inhibitors of cell proliferation. A *NRAS* or a *BRAF* mutation produces a continued

activation of the mitogen-activated protein kinase (MEK) and the extracellular signal regulated kinase (ERK) (Figure 2).

*BRAF* mutations occurs early and are missense mutations (point mutations in which a single nucleotide change, results in a codon that codes for a different amino acid). The frequency of *BRAF* mutations is around 50% in cutaneous melanoma and around 10 % in mucosal melanoma [21]. The most frequent mutations result in the substitution of a valine at codon 600, with a glutamate (V600E), lysine (V600K) or arginine (V600R). *NRAS* mutations are found in approximately 25% of the patients and *NRAS* Q61R or Q61K are the most frequent amino acid changes resulting from these mutations [22].

*NF1* inactivating mutations are present in approximately 14% of all melanoma and this makes the NF-1 protein unable to inhibit the GTPase that activates *NRAS* [22]. The fourth most common setting of mutations is the triple negative, where driver mutations in *BRAF*, *NRAS* or *NF1* are lacking. This pattern is instead usually associated with the genetic modifications of the receptor tyrosine kinase (KIT) or the G protein subunit alpha Q (GNAQ) coding regions.



**Figure 2.** The MAPK pathway is a critical driver pathway in melanoma. Here are showed the most common mutations associated *BRAF*, *NRAS*, and *NF-1*. Multiple target therapies are now available targeting both *BRAF* and *MEK*. Figure adopted from: *The Biology and Therapeutic Approach to BRAF-Mutant Cutaneous Melanoma*. Wood K., Luke J. *The American Journal of Haematology /Oncology. AJHO.* 2017;13(1):4-10

## 1.6. Staging

Melanoma is staged according to the American Joint Committee on Cancer (AJCC) staging manual, currently the 8th edition. This system includes characteristics of primary tumour (T), status of lymph nodes and non-nodal locoregional sites (N), as well as any distant metastasis (M) status (Table 1).

Clinical staging				Pathologic staging			
<b>Stage 0</b>	Tis	N0	M0	<b>Stage 0</b>	Tis	N0	M0
<b>Stage IA</b>	T1a	N0	M0	<b>Stage IA</b>	T1a	N0	M0
<b>Stage IB</b>	T1b	--	--	<b>Stage IB</b>	T1b	--	--
	T2a	--	--		T2a	--	--
<b>Stage IIA</b>	T2b	N0	M0	<b>Stage IIA</b>	T2b	N0	M0
<b>Stage IIB</b>	T3a	--	--	<b>Stage IIB</b>	T3a	--	--
	T3b	--	--		T3b	--	--
	T4a	--	--		T4a	--	--
<b>Stage IIC</b>	T4b	--	--	<b>Stage IIC</b>	T4b		
<b>Stage III</b>	Any T	≥N1	M0	<b>Stage IIIA</b>	T1-2a	N1a	M0
	--	--	--	<b>Stage IIIB</b>	T1-2a	N2a	--
	--	--	--		T0	N1b-c	--
	--	--	--		T1-2a	N1b-c	--
	--	--	--		T1-2a	N2b	--
	--	--	--	<b>Stage IIIC</b>	T2b-3a	N1a-2b	--
	--	--	--		T0	N2b-c	M0
	--	--	--		T0	N3b-c	--
	--	--	--		T1a-3a	N2c-3c	--
	--	--	--		T3b-4a	Any N	--
	--	--	--		T4b	N1a-2c	--
	--	--	--	<b>Stage IIID</b>	T4b	N3a-c	M0
	--	--	--				
<b>Stage IV</b>	Any T	Any N	M1	<b>IV</b>	Any T	Any N	M1

**Table 1.** AJCC 8th edition on Melanoma clinical and pathological classification.

### 1.6.1. Primary tumour status (T)

The primary tumour status contains the two most important characteristics, Breslow thickness and ulceration. Melanomas with a thickness between 1.0 and 2.0 mm are classified as T2; T3 identifies lesions between 2.0 and 4.0 mm and T4 are melanomas thicker than 4.00 mm. Each class is further being divided into “a” or “b” depending on the absence or presence of ulceration. The only exception is T1 in which T1a is a melanoma less than 0.8 mm without ulceration and T1b that includes both an ulcerated lesion less than 0.8 mm or a lesion between 0.8-1.0 mm independently of ulceration.

When a primary melanoma has disappeared due to regression or when data related to thickness and ulceration is missing this is defined

as. In the specific case when the primary lesion was never identified, tumour status is defined as a T0.

#### *1.6.2. Lymph node and non-nodal locoregional sites status (N)*

In this category both the status of the regional lymph nodes as well as the status of non-nodal locoregional sites (satellites or in-transit metastases) are included. The regional draining basins are divided into three main N categories based on the number of lymph nodes with metastases, N1 when one positive node, N2 when two or three positive nodes are present and N3 when more than three lymph nodes are present.

Each category is further divided into “a”, “b” and “c”, where “a” is defined as “clinically occult” and includes positive sentinel nodes and “b” is defined as “clinically detected” nodes and includes nodes detected by clinical, radiological or ultrasound examination. The “c” category is due to the presence of microsatellite, satellite, or in-transit metastases. These are all signs of intra-lymphatic or angiotrophic invasion that defines a more severe metastatic disease, even if in the presence of a smaller number of involved nodes. Microsatellites are small tumour deposits in close proximity of the primary tumour, satellites are defined as tumours within 2 cm from the primary tumour and in-transit metastasis defined as tumours located beyond 2 cm of the primary site and before the first regional draining basin.

When there are no signs of metastatic disease in regional lymph nodes this is classified as N0. When the lymph node status is unknown this is classified as Nx.

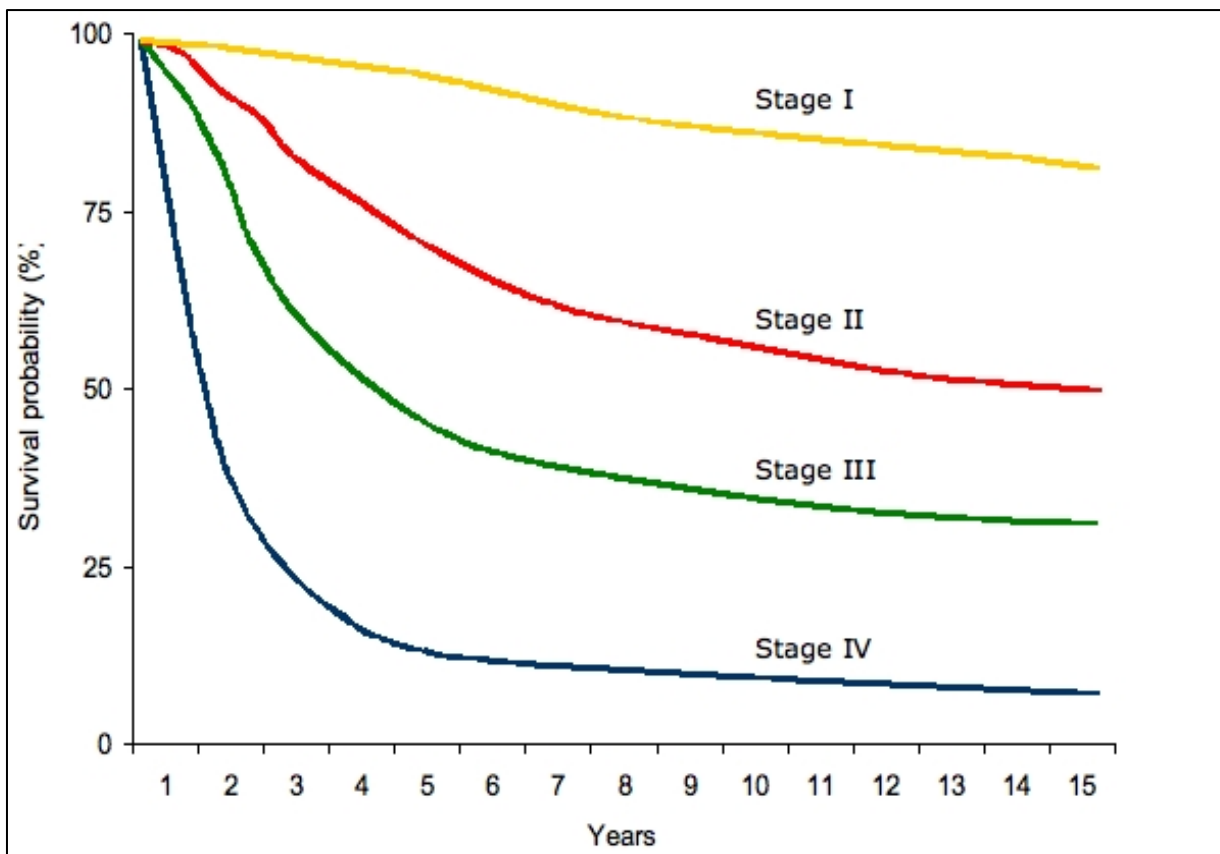
#### *1.6.3. Distant metastasis status (M)*

The M category classifies patients based on the absence (M0) or presence (M1) of distant metastatic disease. The M1 class is further divided into four levels, “a” for cutaneous and subcutaneous metastases or metastases in distant lymph nodes, “b” for lung metastasis, “c” for all other visceral sites and “d” for brain metastasis. The level of serum lactate dehydrogenase (LDH) is reported as a third classifier and is reported as (0) when normal and (1) when increased.

#### 1.6.4. Stages

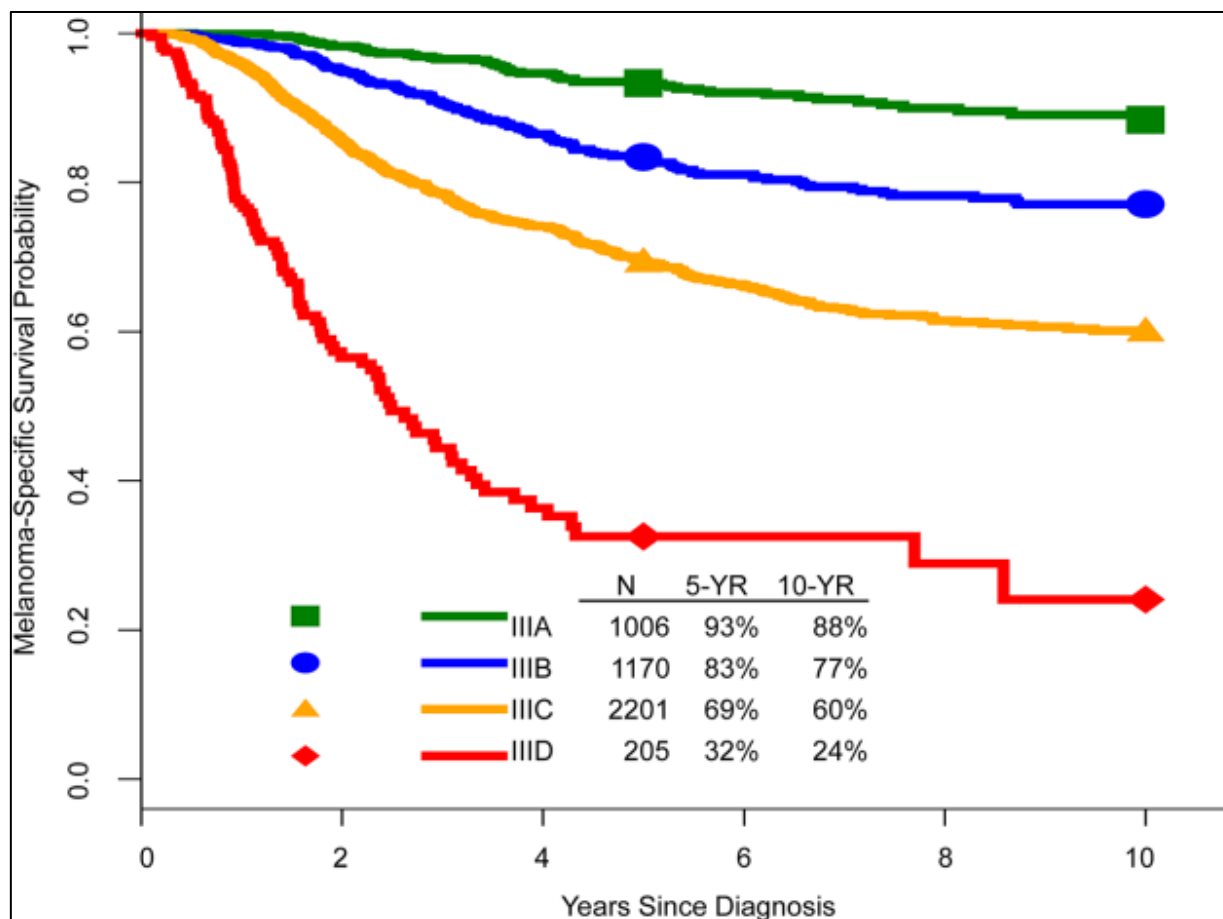
According to the TNM status, patients are then categorized into four different stages (Table 1). Clinical staging is derived by the merge of data from the pathologist analysis related to the primary melanoma and the evaluation of clinical and radiologic exams regarding regional and distant metastases. Pathologic staging includes the information retrieved from a SNB or completion lymph node dissection (CLND).

The four stages present a significant difference in survival (Figure 3), where the 15-year overall survival is approximately 80% for patients in stage I compared to approximately 30% for stage III and 5% for stage IV disease.



**Figure 3.** Figure adopted with permission from the website Melanoma Molecular Maps Projects ([http://www.mmmp.org/MMMP/import.mmmp?page=sc\\_adv.mmmp](http://www.mmmp.org/MMMP/import.mmmp?page=sc_adv.mmmp)).

There is also a substantial difference in melanoma specific survival (MSS) rates within the Stage III, with the 10-year survival spanning between 90% and 20% (Figure 4).



**Figure 4.** Survival between different stages of melanoma I-IV. Figure adopted from Melanoma staging: Evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J Clin. 2017 Nov;67(6):472-492.

## 1.7. Surgical treatments

The removal of a primary pigmented skin lesion, the diagnostic excision, has both curative and prognostic value. If the diagnosis is melanoma, several factors influence both prognosis and the therapeutic strategy. In case of a melanoma *in situ*, no other surgery is needed if the excision was made with at least 5 mm margin. If the diagnosis is an invasive melanoma, a wide local excision is recommended.

### 1.7.1. Wide local excision

The wide local excision related to (WLE) is the surgical excision of the previous scar from the diagnostic excision with a margin on the sides and extending the excision down to the next anatomical layer, usually the muscular fascia, with the aim to remove any potential satellite lesions or micro-metastasis spreading from the primary melanoma. In Sweden, the recommended margin of resection from the primary

tumour scar is 1 cm in case of Breslow thickness  $\leq 1.0$  mm or 2 cm if thicker than 1.0 mm [16].

The complications after a WLE includes tissue defects, increased hospital stay, prolonged rehabilitation, risk of chronic pain and need for reconstructive surgery. The current recommended margins have been defined by international randomized trials slowly shrinking the margins from 5 cm to 1-2 cm. Currently there is a new randomized trial evaluating if 1 cm margin is sufficient also for thicker melanomas, the MelMart trial [23].

#### *1.7.2. Sentinel node biopsy and completion lymph node dissection*

The sentinel node (SN) is defined as the first node of a lymphatic basin receiving the lymphatic drainage from a specific anatomic area [24]. Sentinel node biopsy (SNB) was first introduced for melanoma by Morton et al. in 1992. The technique is based on lymphatic tracing using a marker which is injected intra-dermally in close proximity to the tumour or the previous scar that is then drained through the lymphatics and is trapped in the first lymph node for this area, the SN. Several markers have been used: blue dye, isotope marked solutions, magnetic compounds and others. The SN is removed surgically and then analysed by the pathologist deciding if there are tumour deposits present. The SN is the most important prognostic factor for patients with melanoma, but the therapeutic effect can be questioned [25].

The MSLT-1 trial studied the SNB technique and randomized patients into either WLE alone, with delayed LND in case of growth of nodal recurrence or WLE and SNB, with immediate completion lymph-node dissection (CLND) for patients with a positive SN. No difference in survival could be shown, but in a highly discussed sub-group analysis (patients with intermediate thickness between 1.2mm and 3.5mm) comparing the SN-positive patients with patients showing late recurrences in the observation arm, the study reported that there was a survival benefit by performing SNB and immediate CLND [26].

To further explore this finding, the MSLT-2 trial randomized patients with positive SN to either CLND or observation of the nodal basin with ultrasound. The final results showed no difference in melanoma-specific survival (MSS), even in the presence of a higher rate of nodal recurrences in the observation group [25]. Another study, the German

DeCOG-trial, with a similar trial design randomizing patients with positive SN to CLND or observation with ultrasound showed no difference in MSS [27].

## **1.8. Regional therapies**

### *1.8.1. Isolated limb perfusion*

Isolated limb perfusion (ILP) was initially described by Oscar Creech et al. in 1958 [28]. The technique is performed by open surgical access to the central venous and arterial blood flow of the limb, which is proximally isolated by a tourniquet and then connected to an extracorporeal perfusion circuit. A high concentration of a chemotherapeutic drug is thereafter circulated through the limb, limiting the systemic side effects of the drug, as local or systemic toxicity.

The first leakage monitoring system was introduced by Stehlin et al. in the early 1960s. The technique was based on the infusion of a radioactive mark into the perfusion circuit, testing its own isolation level by a scintillation probe fixed over the heart to detect any track of radioactivity in the systemic circulation [29].

ILP has proven to be effective and safe with a high overall response rate (ORR) of 90% and low rates of regional and systemic toxicity rates [30]. In patients not responding completely, or having a recurrence after a previous ILP, a repeated procedure (re-ILP) is possible [31-33].

### *1.8.2. Isolated limb infusion*

Isolated limb infusion (ILI) was described by John Thompson et al. in 1996. The procedure is an alternative to ILP and is based on the percutaneous placement of arterial and venous catheters passing through the contralateral groin without a surgical isolation of the vessels. After a radiological evaluation of the catheters position, a proximal tourniquet is placed on the affected limb. Using a high-flow three-way stopcock syringe, melphalan is infused manually during 20-30 minutes [34]. No randomized trials comparing ILI and ILP have been performed. A retrospective study including 203 patients, of whom 94 underwent ILI and 109 ILP, showed an ORR of 53 % for ILI and 80 % for ILP [35].



### *1.8.3. Other regional therapies*

Electrochemotherapy (ECT) is a locoregional technique based on a selective permeability produced by short electric pulses that open ionic membrane channels otherwise impermeable to the chemotherapeutic agent bleomycin [36]. A recent prospective cohort study showed how this technique can achieve an ORR of 78%, including a 54% CR rate [37].

Talimogene laherparepvec (TVEC) is a local injection therapy for unresectable metastatic melanoma lesions (stage IIIB–IVM1a) approved in a phase III trial (OPTiM) [38]. The mechanism of action is based on the injection of a genetically modified herpes simplex virus type 1 (HSV1) that selectively replicate inside tumour cells and produce granulocyte–macrophage colony-stimulating factor (GM-CSF). The selective cellular lysis releases cancer-related antigens that is phagocytized by antigen-presenting-cells activated by the GM-CSF [39, 40]. The OPTiM trial reported an ORR of 32% with a CR of 15% for the injected lesions, and an ORR of 18% with a CR of 6% for the non-injected lesions [41].

PV-10 is another local injection therapy containing a 10% solution consisting of the xanthine dye Rose Bengal in a sterile saline. Rose Bengal was initially utilised to diagnose ophthalmologic damages and liver cancer; its mechanism of action seems to be due to the xanthine dye which generates reactive oxygen species causing a phototoxic damage. Rose Bengal results both in a direct effect on injected lesions by a phototoxic reaction and an indirect action on untreated lesions by increasing the up-take of cancer antigens by dendritic cells leading to an activation of T lymphocytes [42]. A recently published multicentric single arm phase II trial including 62 unresectable stage III and 18 stage IV patients reported an ORR of 51% with a CR of 26% [43].

## **1.9. Systemic treatments**

The medical management of melanoma has undergone remarkable changes during the last ten years. Nowadays there are several options including traditional chemotherapy, targeted therapies and checkpoint inhibitors.

### *1.9.1. Chemotherapy*

Chemotherapy, most frequently based on dacarbazine (DTIC) and temozolomide, is currently used as a second or a third line treatment for patients in the metastatic setting who are not responding to targeted or immuno-therapies. These drugs are working with the classical mechanism based on cytotoxic action against rapidly proliferating cells [44].

DTIC was first approved in the 1970s with an ORR of 10-20%, however with very few CR [45]. The DFS was approximately 3-6 months with limited number of patients with durable remission [46]. There was no impact on OS for patients treated with DTIC. Temozolomide did not show any advantage in response or survival compared with DTIC, but are orally available and active also at brain metastases [47].

### *1.9.2. Targeted therapies*

At the moment the most used targeted therapies are BRAF and MEK inhibitors. BRAF inhibitors (e.g. vemurafenib or dabrafenib) are therapies able to lock the ATP binding site of the MAP kinase, freezing the protein at an inactive state [48]. Vemurafenib showed in a randomised phase III trial (the BRIM3 trial) an increased OS from 9.7 to 13.6 months compared to DTIC [49].

The MEK inhibitors (e.g. trametinib and cobimetinib) action is intended to block the MEK protein in the MAPK pathway. Given together with BRAF inhibitors this reduce resistance and the phase III trial COMBI-d demonstrated an improved OS with the combination of dabrafenib and trametinib versus dabrafenib only in BRAF V600E/K-mutant metastatic melanoma with a 3-year OS of 44% versus 32% [50]. New agents have recently been tested as mono or combination therapy with promising results. The COLUMBUS study showed that combined therapy with encorafenib and binimetinib has a better tolerability when compared to other treatments and also better response rates [51, 52].

### 1.9.3. Immunotherapy

Modern immunotherapy is based on recombinant antibodies against checkpoint proteins on the cytotoxic T cells, the natural defenders of the adaptive immune system.

Ipilimumab is an antibody against the inhibitory checkpoint CTLA-4 (cytotoxic T-lymphocyte associated antigen-4). When blocking its inhibitory activity, CTLA-4 on the surface of the T cells can no longer compete with the stimulatory co-receptor CD28 for binding to the B7 receptor on the surface of antigen presenting cell [53]. A randomized trial assigned patients in a 3:1:1 ratio to receive a cancer vaccine based on glycoprotein 100 (gp100) alone, gp100 with ipilimumab or ipilimumab alone. The median OS were 6.4 months, 10.0 months and 10.1 months, respectively, showing a significant increase in survival for ipilimumab. The ORR was 11% in the ipilimumab group vs. 1.5% in the gp100 group [54].

Nivolumab and pembrolizumab are examples of two monoclonal antibodies against PD-1 (programmed cell death 1), a co-inhibitory molecule on T cells which, when in contact with ligands (PD-L1, PD-L2) present on tumour cells and stroma cell, inactivate the T cells. In a randomized trial (KEYNOTE-006) pembrolizumab compared to ipilimumab showed a 2-year OS of 55% versus 43% [55]. For nivolumab a randomized trial (CheckMate 066) showed an OS rate at 1 year of 73% in the nivolumab group compared to 42% in the DTIC group [56].

### 1.10. Mouse models in melanoma

Three kinds of mouse models are most frequently used in melanoma research: syngeneic transplants, genetically engineering mouse models (GEMM) and xenograft models.

Syngeneic transplants models are based on the transplantation of tumour cells that have first developed in mice, often because of carcinogen exposure. A frequently used example of this model is C57BL/6 mice (e.g. B16-F10) for study of metastases that was developed by Fidler [57].

The first example of genetic engineered mouse model (GEMM) was “the oncomouse” in 1987 [58]. It was a GEMM carrying a specific

transgenic activated oncogene (v-HRas) under control of a mammary specific promoter (MMTV), resulting in tumorigenic proliferation. This step was an epochal passage that demonstrated in an unequivocal way that the expression of oncogenes in normal cells could induce the formation of tumours. A second model supporting this theory was the tumour suppressor gene *TP53* knockout mice reproducing the condition of loss or inactivation of tumour suppressors [59].

In melanoma GEMMs could be grouped in two categories: one created by using transgenes or virus carrying oncogenes like *BRAF*/*NRAS*, and the other based on knock-out mice to target tumour suppressor genes in combination with *BRAF* activation. Here Cre recombinase is used to delete a stop cassette upstream of the mutated *BRAF* allele and simultaneously deleting tumour suppressor gene *PTEN*. Cre is in this model expressed specifically in melanocytes [60].

The discovery of CRISPR/Cas9 makes the cancer modelling in mice more rapid and flexible with the possibility to insert point mutations, translocations or gene deletions. These efforts led to the creation of new non-germline models [61].

A limitation of GEMMs is that they are not human and that they have low-mutational load. This can be overcome by using xenograft models, the third type of mouse model where the tumour is from human cells. One xenograft model is based on either cell derived xenografts (CDX) or patient derived xenografts (PDX) [62]. To generate xenografts, immunocompromised mice are needed, since the tumour would otherwise be rejected. The first immunocompromised model was the athymic Nude mice with an immune system not able to recognize the human cells as foreign. Then several type of immunocompromised mouse models were generated. Nude mice do not have functioning T cells, but working B and NK cells.

The “Non obese diabetic/severe combined immunodeficiency” (NOD-SCID) mice lack functioning B and T cells but have very few NK cells. NOG/NSG mice are characterized by a further knockout mutation of the IL-2 gamma-chain receptor producing inactive B, T and NK cells. In our studies we used NOG mouse (e.g. NOD/Shi-scid IL-2R<sup>γ</sup>null)

which at the moment has the highest efficiency of engraftment of human cells. This model lacks functional B, T and NK cells as well as having macrophage and dendritic cell dysfunction [63].

## 2. AIMS OF THE THESIS

The overall aim of the thesis is to analyse the outcomes of four studies representing the ongoing evolution in clinical and experimental surgery to improve outcome for patients with cutaneous melanoma.

The specific aims are:

- *To investigate predictive factors for positive sentinel node (SN) and non-SN, as well as prognostic factors for melanoma-specific survival.*
- *To investigate the outcome after repeated isolated limb perfusion in terms of safety and efficacy.*
- *To evaluate the role of BRAF mutational status as a predictive factor for response after ILP.*
- *To establish a biobank with viable tissue and cells from patients with melanoma and generate patient-derived xenografts (PDX) to model cancer immunotherapy.*

### **3. METHODS**

#### **3.1. Study populations**

##### **Paper I**

Between 2000 and 2013, 769 consecutive patients with cutaneous melanoma treated with SNB at Sahlgrenska University Hospital were included from a prospectively kept database. Data concerning the SN tumour load, evaluating the largest tumour deposit, and defined this as low if  $\leq 1\text{mm}$  or high when  $>1\text{mm}$ . The median follow-up time was 55 months.

##### **Paper II**

Between 2001 and 2015, 290 consecutive patients treated with 380 ILPs were included from a prospectively kept database at Sahlgrenska University Hospital. Ninety of these were re-ILPs, 68 patients were perfused two times, 16 patients three times, 4 patients four times and 2 patients received the treatment 5 times.

##### **Paper III**

Between 2012 and 2017, 111 consecutive patients with melanoma in-transit metastases treated with first-time ILP at Sahlgrenska University Hospital were included from a prospectively kept database. Data for both response and BRAF-mutational status were available for 98 patients (88%) and these patients were included into the final analysis. There were 32 patients with a BRAF V600E/K mutation (33%) and 66 patients having a BRAF wt (66%), equally distributed between males and females.

##### **Paper IV**

From 21 patients between 2013 and 2018 at Sahlgrenska University Hospital with metastatic melanoma cryopreserved biopsies were stored in a biobank. These tumours were transplanted into either NOG mice or hIL2-NOG mice. Clinical follow-up data (treatment, response, tumour markers, radiology and survival) were retrieved from medical records. An additional patient was also followed with real-time biobanking involving biopsy transplantation and TIL generation.

### **3.2. Data retrieval**

Data concerning response and progress was collected from a prospectively kept database at the Department of Surgery, Sahlgrenska University Hospital. Data concerning survival and cause of death was recovered from the Swedish National Cause of Death Register (Swedish National Board of Health and Welfare).

### **3.3. Sentinel node biopsy**

Following a diagnostic excision of the primary melanoma (Stage 1-2) patients underwent WLE and SNB. SNB was performed using the combination of a lympho-scintigraphy, blue dye injection and the use of an intraoperative gamma probe. The day before surgery 80Mbq technetium-99-nanocolloid was injected around the primary tumour site. After two hours images were acquired to track the main lymphatic drainage and to find the first draining lymph node. Patent Blue Violet (Patent Blue V 2.5%; Guerbet, Aulnay-sous-Bois, France) was injected immediately before the intervention in the operating theatre intracutaneously in four points around the scar of the primary lesion. The removed lymph nodes were sent for pathological examination using haematoxylin and eosin staining. Starting in May 2013, also immunohistochemistry using the markers S-100, Melan-A and HMB-45 were used routinely. Patients with a positive SN were planned for a CLND.

### **3.4. Isolated limb perfusion**

The vascular system of the treated limb was isolated by cannulation and clamping of the major artery and vein, which were then connected to an oxygenated extracorporeal circuit. The remaining collateral vessels were compressed with a proximal inflatable tourniquet (Zimmer disposable tourniquet) or an Esmarch bandage.

For first-time ILPs, melphalan (M-ILP) (Alkeran®, GlaxoSmithKline Pharmaceuticals, Research Triangle Park, NC) was used. The addition of tumour necrosis factor-alpha (TNF-alpha, Beromun®, Boehringer Ingelheim, Germany) (TM-ILP) was considered primarily for bulky melanomas (tumours with a diameter larger than 30 mm) but also for re-ILPs.



In M-ILP, the dose of melphalan was calculated according to the limb volume using 13mg/L for upper limb and 10 mg/L for lower limb perfusions. Before 2012, melphalan was given as three bolus doses, with 50% of the total dose administered initially and the remaining 50% administered in two equivalent doses at 30-minute intervals, with a total perfusion time of 90 minutes. In 2012 the administration of melphalan was changed to a 20 minutes infusion into the perfusion circuit and a total perfusion time of 60 minutes [64].

In patients receiving TM-ILP, a bolus dose of TNF-alpha was injected into the perfusion circuit (3 mg for upper limb and 4 mg for lower limb), provided that the limb tissue temperature had reached 38°C. Thirty minutes later melphalan was then administered during a 20 minutes infusion. The total perfusion time was 90 minutes. All treatments were performed under mild hyperthermia (39-40°C). After perfusion, the limb was rinsed with at least 1–2 L (upper limb) or 3–4 L (lower limb) of Ringer's solution (Ringer Acetate, Baxter, Sweden). No adjustments in perfusion characteristics were made for re-ILPs.

During the procedure, continuous leakage monitoring was performed with a precordial scintillation probe (MedicView, Gothenburg, Sweden) to detect and measure leakage of technetium-99m labelled human serum albumin (Vasculosis, Cis Bio, France) injected into the perfusion circuit.

### **3.5. Response evaluation**

Response was evaluated according to the WHO criteria at 3 months from treatment [65]. A complete response (CR) was defined as the disappearance of all lesions. Partial response (PR) was categorized a reduction of more than 50% of the total tumour burden. Progressive disease (PD) was identified by a growth in volume of more than 25% of the existing lesions or the appearance of new ones. Stable disease (SD) describe the condition in which criteria for CR, PR or PD were not fulfilled.

### **3.6. Evaluation of toxicity and complications**

Local toxicity was monitored and evaluated by a physician up to three months after ILP and graded as the worst toxicity during that time according to the Wieberdink classification; grade I (no reaction), grade

II (erythema or swelling), grade III (major erythema, swelling or blistering), grade IV (extensive epidermolysis and/or damage to deep tissues, causing final functional disorders; risk or manifest compartment syndrome) and grade V (reaction that may necessitate amputation) [66].

Complications within 30 days post-operatively were graded according to the Clavien-Dindo classification [67].

### **3.7. Statistical methods**

Univariate and multivariate logistic regression analysis using the Enter method were performed to find predictive factors for SN and non-sentinel node (NSN) positivity after surgery and for response and toxicity after ILP. False negative rate (FNR) was defined as the ratio between false negative cases and the total number of false negative and true positives.

Disease-free survival (DFS) was defined as the time between surgery and recurrence as detected with either clinical or radiological examination. Survival was calculated from surgery or first ILP to death, either from melanoma (melanoma-specific survival, MSS) or including other causes (overall survival, OS). The Kaplan-Meier method was used to perform time-to-event curves, then compared with the log rank test. Cox proportional hazard regression analysis using the enter method was used for multivariate analysis.

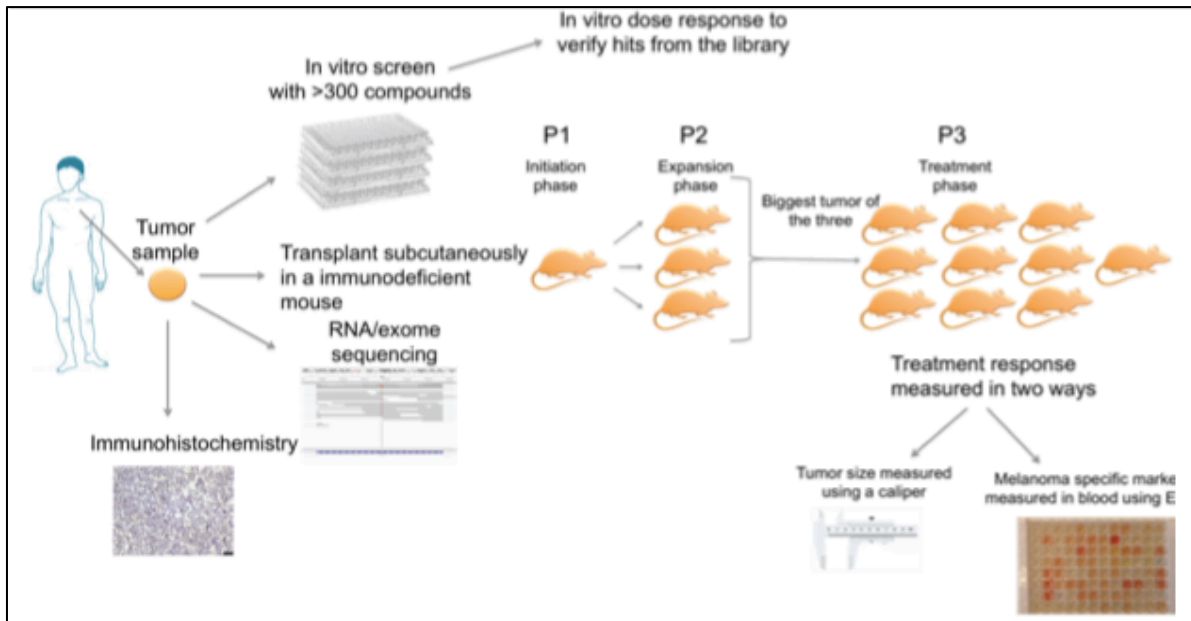
A p-value of  $<0.05$  was considered significant. Missing data were excluded from the analysis. IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, New York, USA) was used for statistical analysis.

### **3.8. Laboratory analysis of BRAF mutational status**

*BRAF* gene mutation analysis uses DNA sequencing to detect mutations in the *BRAF* oncogene. The test is used routinely in Sweden for patients with melanoma stage III and IV, and the analysis is performed by six different clinical molecular pathology laboratories in the different Swedish regions. Data concerning BRAF-mutational status was retrieved from the clinical molecular pathology departments of the referring hospitals.

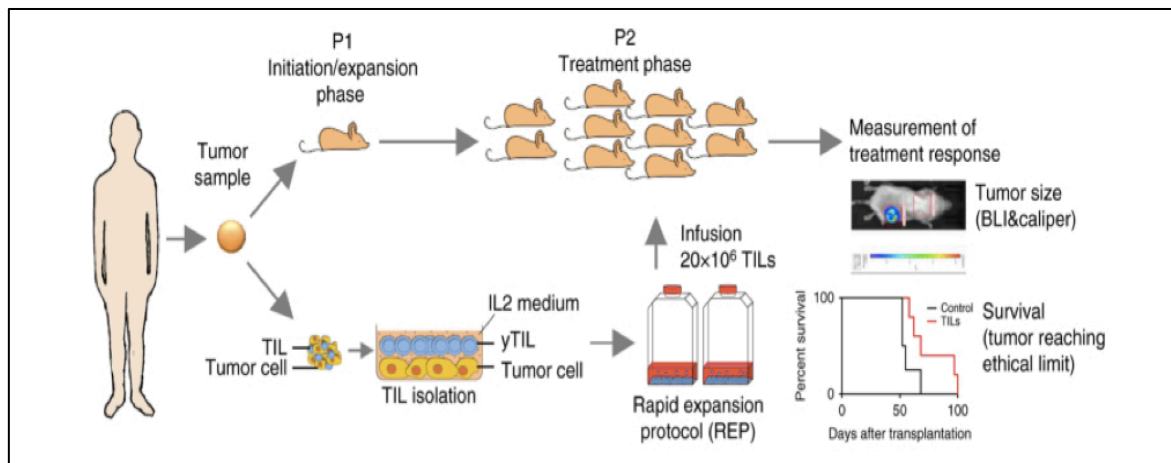
### 3.9. Patient-derived xenografts

Following informed consent (Ethics approvals #288-12 and #44-18), patient-derived xenografts (PDX) were created by subcutaneous injections of tumour cells from patients with melanoma into NOG mice (Figure 5).



**Figure 5.** Patient derived xenograft model standard procedure. Figure adopted from: Einarsdottir, B.O., et al., Melanoma patient-derived xenografts accurately model the disease and develop fast enough to guide treatment decisions. *Oncotarget*, 2014. 5(20): p. 9609-18.

This model had the limitation of not modelling the interaction between tumour cells and the immune system, since NOG mice are deficient in number and function of B, T and dendritic cells, and deficient in function and reduced numbers of natural killer cells. Another model called PDXv2 was developed; in this model both tumour cells and the tumour infiltrating lymphocytes (TILs) expanded in cell culture were injected into the mice (Figure 6) [68]. For this model to support viability of human T cells, a transgenic NOG mouse expressing human IL-2 (hIL2-NOG). We also transplanted biopsies from patients directly into NOG or hIL2-NOG mice.



**Figure 6.** In this schema is shown the isolation process from a tumour sample of both cancer cells and TILs, then respectively injected in mice and expanded in lab culture. Figure adopted from: Jespersen H et al. Clinical responses to adoptive T-cell transfer can be modelled in an autologous immune-humanized mouse model. *Nat Commun.* 2017 Sep 27;8(1):707.

For both PDXv2 and biopsy transplantation models, tumour initiation was performed by a 1:1 ratio mix of cell suspensions and Matrigel, then injected in the subcutaneous tissue of 8-24 weeks-old NOG mice or hIL2-NOG mice (from Taconic or own breeding colony). Twice a week tumour growth was checked by caliper measurement. The tumour volumes were calculated using the formula  $(\text{length} \times \text{width}^2)/2$ . The mouse was sacrificed when a tumour reached more than 10 mm in the base, or it was registered a loss of weight higher than 20%.

In PDXv2, tumours growing in NOG mice were serially transplanted into hIL2-NOG mice. Twenty million TILs were injected either when tumours had grown or five days after tumour implantation.

### 3.10. Production of TILs

Tumour biopsies were cut into small pieces, and TILs were produced by culturing bioptic samples in 24-well plates in TIL medium (RPMI, 10% human serum and 6000 U/ml IL-2 from Peprotech). These young TIL (yTIL) cultures were either cryopreserved or used in a rapid expansion protocol (REP) for in vivo experiments. For REP, yTILs were mixed with irradiated feeders from allogenic donors (Sahlgrenska Blood Depository) and cultured in REP medium (50% RPMI, 40% AIM V, 10 % human serum, 6000 U/ml IL-2 and 30 ng/ml anti-CD3 antibody (OKT3 antibody).

### **3.11. Genomics**

DNA and RNA were extracted from tumours and TILs using a kit (Qiagen). Exome and RNA-seq was performed in the lab (GeneCoreSU) and raw reads were aligned to the human genome. TILs were used as normal controls for exome sequencing data.

## 4. RESULTS AND DISCUSSION

### 4.1. Paper I

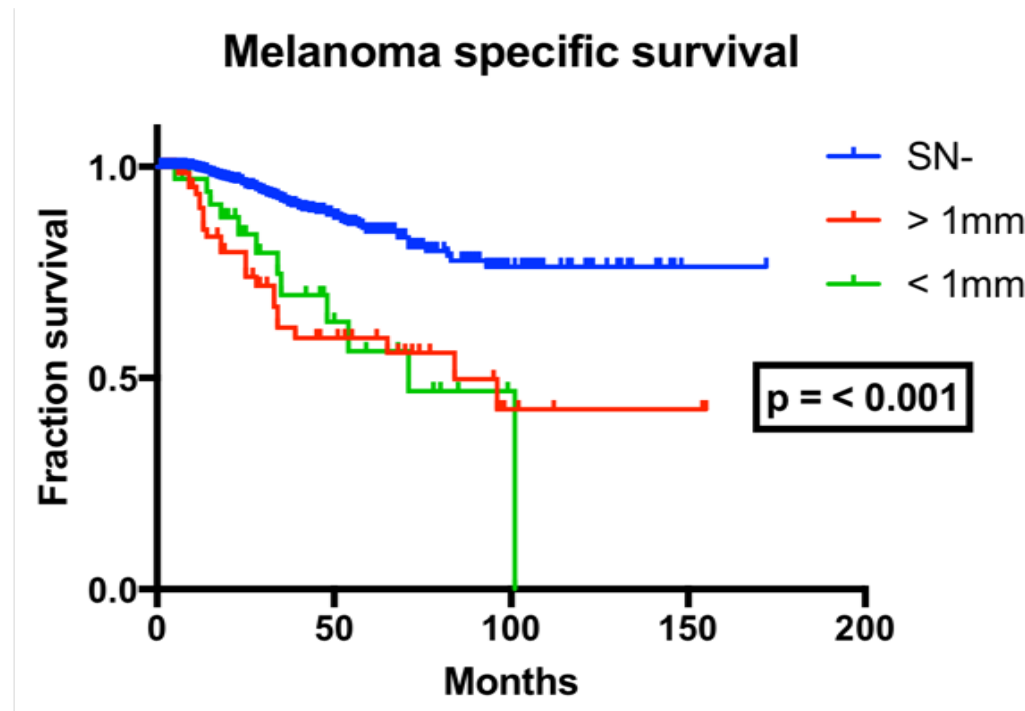
This study includes the outcome of all SNB performed at Sahlgrenska University Hospital between 2000 and 2013. During this period the number of procedures per year increased significantly and SNB became a routine management for T1b-T4 melanomas. Across the study period the rate of positive SN was 14%, which is comparable to other reports ranging between 13% and 31% [69, 70].

The FNR was 20% for the entire period, comparable with previous studies ranging between 9% and 21% [71, 72], reducing progressively during the years to settle around 17% in the last time period [73-75]. This confirms the existence of a learning curve in performing a SNB favouring the centralization of specialist surgery within dedicated cancer centres [76]. The only independent predictive factor for a positive SN found in our series was Breslow thickness. It was not possible to identify already known predictive factors such as ulceration, age or tumour site as predictive for SN positivity. Other known predictive factors with less importance, such as regression, lympho-vascular invasion and mitosis of the primary tumour was not included in the analysis [19, 26, 73, 77-79].

A positive non-sentinel node (NSN) was detected in approximately 20% of the patients with a positive SN that underwent CLND. Independent predictive factors for positive NSN were reported and extensively analysed by Madu et al. They found that the largest diameter in SN metastasis was the most important predictive factor [80]. Our study could not confirm this observation, possibly due to the low number of patients with positive NSN.

In terms of survival the most important prognostic factors were shown to be SN-status, Breslow thickness and ulceration. These findings confirm the importance of these parameters as the most important prognostic factors for melanoma according to the current AJCC classification [16, 81]. The 5-year MSS was 81% and there was no difference in MSS related to the tumour load in SN (Figure 7), which is different from other previous reports [82-84]. Potentially this could be due to the differences in the pathological analysis of the SN, where

more information concerning the tumour load could have been obtained by e.g. the Rotterdam criteria [84].



**Figure 7.** Kaplan Meier survival curves in patients that underwent SNB.

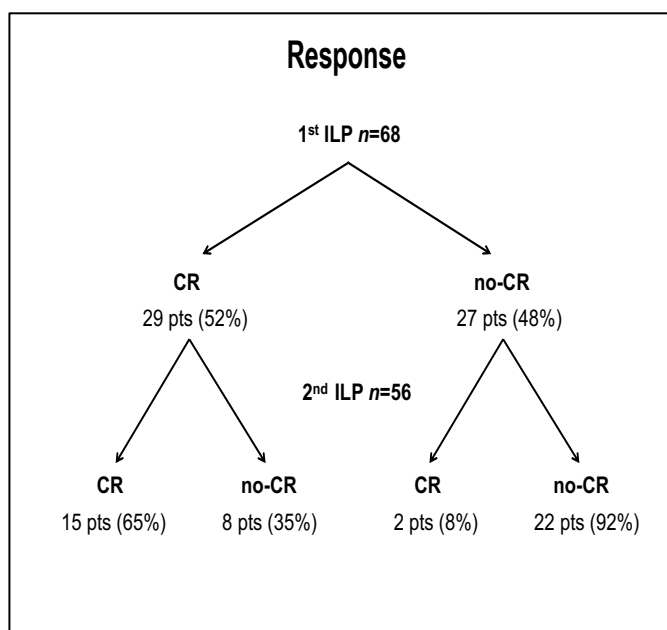
## 4.2. Paper II

Clinical response was analysed in 380 ILPs and the outcomes were evaluated dividing the population into three groups: patients who underwent only one ILP, patients treated twice and patients who received three to five ILPs. The ORR registered for the three groups was 83%, 80% and 68% with a CR rate of 60%, 41% and 59%.

In this study the response for first-time ILPs was comparable to previous results from our own institution [64, 85] and also to previous reports from other units [30]. On the other hand, when only analysing the re-ILPs, we discovered that our response rates were in the lower range with a CR rate of 52%, compared to other major studies on re-ILP reporting a CR rate between 62% and 76% [31-33]. This result is probably due to a selection bias, where other institutions mainly perform re-ILPs in patients with recurrences following complete or near-complete response after previous ILP [31-33], while in our

series, many re-ILPs were on patients not responding after the first ILP (Figure 8).

In this study 2/3 of the patients developed a grade I/II toxicity independently of the number of ILPs; these findings are similar to those of other series reporting grade I/II reactions between 66 and 73% [30-33]. Severe reactions such as grade IV toxicity were 7% in this series, which is higher than other reports ranging between 0% and 5% [30-33]. A potential explanation could be a more accurate long-term follow-up of the patients, where any deficit in limb sensitivity or motor function was reported as a persistent grade IV toxicity. In our practice re-ILP were performed using previous incisions, making the surgical access more challenging, but without an increase in complications, when compared to first-time ILPs.



**Figure 8.** Patients response to ILP, showing the higher number of CR after a second ILPs in patients who had a CR already at first ILP.

The use of TNF-alpha in re-ILPs have been highly discussed. In the group of first-time ILPs, there was a difference in response after M-ILP and after TM-ILP, with a CR rate of 63% and 33% respectively. This finding is probably due to the use of TNF-alpha only for bulky tumours, where the tumour load is a negative predictive factor for obtaining a full response. In the re-ILPs, TNF-alpha was not used in 17 patients and in this group both the OR and CR rates were lower, however not statistically significant in neither univariate nor multivariate analyses. From the current results it was not possible to draw any definitive conclusions about the role of TNF-alpha in the re-ILP.

The overall survival time was 34 months, 41 months and 93 months, respectively for patients who underwent only one ILP, patients treated twice and patients who received 3-5 ILPs (p=0.02). The OS was significantly higher in patients receiving repeated ILPs. This is



probably explained by a selection bias and not as a consequence of the ILP treatment itself. Patients with longer survival are more prone to the risk of loco-regional relapse and they consequently have a higher risk of being treated with a re-ILP.

### 4.3. Paper III

In this study response and BRAF-mutational status were studied in 98 patients treated with a first-time ILP. The ORR was 69% for BRAF V600E/K and 77% for BRAF wt ( $p=0.36$ ). The CR rate for BRAF V600E/K and for BRAF wt patients was 47% and 52%, respectively ( $p=0.67$ ). At univariate analysis, only stage and tumour size were significant predictive factors for a CR, but in the multivariate logistic regression analysis no independent predictive factor was identified (Table 2). Taken together, the BRAF status do not influence the response rates after ILP.

	<i>Univariate analysis</i>			<i>Multivariate analysis</i>		
	OR	95% CI	p-value	OR	95% CI	p-value
Age (year)	1.0	0.9-1.1	0.4	1.0	0.9-1.0	0.6
Gender						
Female* vs. Male	0.7	0.3-1.5	0.3	0.6	0.2-1.4	0.2
Stage						
N2c* vs. N3	10.4	1.2-87.5	<b>0.03</b>	1.7	0.6-4.6	0.3
N2c* vs. M1	13.0	1.5-111.8	<b>0.02</b>	0.2	0.1-2.5	0.2
Numbers of tumors						
≤ 10* vs. > 10	0.5	0.2-1.1	0.1	0.6	0.2-1.6	0.3
Tumor size						
≤ 30 mm* vs. > 30 mm	0.2	0.1-0.8	<b>0.02</b>	0.4	0.8-1.7	0.2
Chemotherapy						
M-ILP* vs. TM-ILP	0.2	0.1-2.0	0.2	0.8	0.1-13.8	0.9
Mutational Status						
BRAF wild type* vs. BRAF V600E/K	0.8	0.3-1.9	0.7	0.6	0.2-1.6	0.3

**Table 2.** Univariate and multivariate logistic regression for complete response.

Our results are concordant with those previously presented by Li et al. on 150 patients, where BRAF status was not found to be predictive for response after ILI [86]. This is however in contrast to Gallagher et al. which reported on 30 patients treated with ILI, where BRAF V600E/K was found as a predictive factor for a poorer response [87].

The median survival was 47 months. For the BRAF V600E/K patients the median survival was not reached, while it was 44 months for the BRAF wt group. The 2-year OS was 51% for the BRAF V600E/K group and 49% for the BRAF wt group. In univariate Cox regression analysis age, sex, stage and tumour size were identified as prognostic factors, however at the multivariate analysis the only independent factors were age and stage. There are conflicting results reported for the prognostic value of BRAF mutational status for survival. An analysis including 197 patients with unresectable stage IIIC and stage IV melanoma demonstrated a worse prognosis for patients carrying a BRAF V600E/K mutation (5.7 months instead of 8.5 months) [88]. This can be compared to a study by Carlino et al. including 192 patients not treated with BRAF-inhibitors, where BRAF mutational status was not identified as a prognostic factor [89].

#### **4.4. Paper IV**

In this study we reported the use of an immune-humanized mouse model as a functional biomarker of response to immunotherapy. In 21 patients it was possible to generate both PDX models and TILs. For each patient we created two animal models by injecting biopsies from patients with melanoma; one in a wild type NOG mouse (NOG/wt) and the other in a mouse expressing human IL2 (hIL2-NOG). We observed that TILs in injected biopsies were not able to stop the tumour growth in NOG mice, while in hIL2-NOG mice TILs from some of the biopsies were able to inhibit cancer growth in some of the models.

There is a lack of methods to predict response to PD-1 inhibitors, and neither mutational load, PD-L1 expression or new markers such as IMPRESS or TIDE are reliable [90, 91]. In this series, the corresponding patients that received systemic immunotherapy, had similar responses as shown in the PDX models, suggesting that the model might be predictive. As an example, a subcutaneous metastasis was surgically removed from a patient with a BRAF

mutated melanoma. When analysing the sample, it was not possible to isolate any TILs. After the surgery the patient was treated with BRAF and MEK inhibitors and responded to therapy. A new biopsy was taken and it was now possible to isolate a large number of TILs, but very few tumour cells. We injected tumour cells and TILs from these two samples in NOG/wt and hIL2-NOG mice. The first sample of the patient showed tumour growth in both models while the second biopsy grew very slow. The patient was switched to anti-PD1 inhibitor and more lesions disappeared. Biopsies during treatment grew in NOG but not in hIL2-NOG.

After two months the patient progressed with new metastases. One superficial metastasis was surgically removed before a re-challenge with BRAF/MEK inhibitors. This biopsy grew both in NOG and hIL2-NOG mice, showing that the last TILs were incapable of killing the cancer cells.

Taken together these data suggest that responses in mice can be used to support clinical decisions. It is indeed plausible that in the future, PDX models will have a chance to guide second and third-line therapies, considering the continuous improvements of clinical efficacy of immunotherapy in correctly selected patients. The obvious limitations of our study and the interpretation are the limited number of samples, and that the biobank was retrospective. However, we were able to show its value supporting the clinical management of patients with metastatic melanoma.

## 5. CONCLUSION

- Breslow thickness was the only independent predictive factor for a positive sentinel node. The prognostic factors for melanoma-specific survival were sentinel node status, Breslow thickness and ulceration. Sentinel node tumour load were not prognostic for survival. We reported a decrease in false negative rate over time with results comparable to other international institutions.
- Patients having a complete response after the first ILP were likely to have the same response at re-ILP without any increase in the risk for local toxicity or complications.
- BRAF mutational status of patients with melanoma in-transit metastases treated with ILP, was not a significant factor for response nor survival.
- The use of advanced PDX models could potentially be used to predict response to immunotherapy in patients with melanoma metastases.

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